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journal homepage: <http://www.ijporlonline.com/>A novel *TECTA* mutation causes ARNSHL

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ABSTRACT

Objective: Autosomal recessive nonsyndromic hearing loss (ARNSHL) is a genetically heterogeneous sensorineural disorder. Alpha-tectorin, which is encoded by the *TECTA* gene, is a non-collagenous component of the tectorial membrane in the inner ear defect of which leads to moderate to severe hearing loss (HL).

Methods: 25 unrelated Iranian multiplex ARNSHL families, negative for *GJB2* mutations, were recruited in this study. Clinical inspections including audiometric and otologic examinations ruled out syndromic forms. Genetic linkage analysis was performed using six short tandem repeat markers closely linked to DFNB21. Haplotype and LOD score analysis were used to confirm possible linkage. All coding exons of *TECTA* were subject to DNA sequencing in the linked family.

Results: A novel homozygous variant (c.734G > A) was found in exon 5 of the *TECTA* gene in one family leading to a nonsense mutation (p.W245X). It co-segregated with HL in the family. This variant was not detected in 50 controls. All affected individuals in the family had moderate to severe HL. It full filled the criteria of a pathogenic variant.

Conclusion: Our data confirms the phenotype-directed genotyping for DFNB21 deafness against the typical profound HL phenotype seen in the most families segregating ARNSHL. We recommend mutation screening of *TECTA* in ARNSHL families segregating moderate to severe HL phenotype.

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1. Introduction

Hearing loss (HL) is the most common sensorineural defects with high genotype and phenotype heterogeneity [1]. According to the World Health Organization, 360,000,000 individuals world wide have different range of disabling HL (90% and 10% this population are adults and children respectively) (www.who.int/) [2]. HL is a multifactorial disorder, predominantly induced by genetic or environmental causes or their interactions [2]. It is estimated that more than half of the cases with congenital sensorineural HL are associated with genetic factors [3]. HL can be inherited as autosomal dominant (DFNA 20%), recessive (DFNB 80%), mitochondrial

maternally, X and Y-linked (2%) [3–5]. Autosomal recessive non-syndromic hearing loss (ARNSHL) is the most frequent form of HL (75–80% of cases), [6]. *TECTA* (DFNB21) is an important gene involved in HL. It comprises 23 exons and 7426 bp of DNA on chromosome 11q22–q24. *TECTA* encodes the 2156 amino acid that is one of the major non-collagenous glycoproteins of the tectorial membrane, a non-cellular matrix overlying the cochlear neuro-epithelium that lies over stereocilia of the hair cells and is critical for the mechanical amplification and transmission of sound [7,8]. Alpha-tectorin is a large modular glycoprotein that contains three types of functional domains: an N-terminus entactin-like domain, four von Willebrand type D (vWFD V1, V2, V3, V4) domains and C-

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terminal zonapellucida (ZP) domain [9], these domains are cross linked to each other by disulfide bridges and interact with another non-collagenous protein [10]. Mutations of various parts of alpha-tectorin leads to deafness at different frequencies and lead to specific genotype–phenotype correlations [11]. Various mutations in this gene have been shown to be responsible for both autosomal dominant (DFNA8/12) [7] and recessive (DFNB21) forms of non-syndromic HL [12]. Studies on different populations have shown that alpha-tectorin is among the top 10 genes responsible for ARNSHL [6,13]. Previous studies have shown that *TECTA* mutations account for about 4.13% of ARNSHL among *GJB2* negative Iranian families [6]. *TECTA* has been shown to be related to moderate to severe audioprofile [14]. Iran has a high frequency (37–38%) of consanguineous or intra group marriage [15]. On the other hand, the presence of families with large multiple-affected deaf individuals has made it suitable for genetic studies [15,16]. In this study, 25 Iranian families with ARNSHL were analyzed for linkage to the DFNB21 locus after excluding mutations in *GJB2*. The specific audioprofile of the patients was also regarded. A single family was linked to DFNB21; DNA sequencing of the *TECTA* gene identified a novel mutation in this gene.

2. Material and methods

2.1. Patient recruitment and audiometric evaluation

Twenty five Iranian families (with 350 individuals that 171 of them were affected) and 50 (100 chromosome) ethnically matched healthy Iranian control individuals which had been ascertained from Khuzestan province, Southwest of Iran. Families were negative for *GJB2* mutations. This study was approved by the institutional review boards of Tehran University of Medical Sciences (No. 150756) and Ahvaz Jundishapur University of Medical Sciences (U-91074) and Shahrekord University of Medical Sciences (1394-01-74-2598). Informed consent was taken from subjects or their parents. Based on the interviews with the adult members of the families, informational questionnaires were filled out and pedigrees were drawn. All the members the families were sampled for further molecular studies.

2.2. Auditory testing

Pure tone audiometry (PTA) with air conduction at frequencies ranging from 250 to 8000 Hz was performed for all affected members of the families according to standard protocols. Audiogram results of all patients were defined using a PTA average at 500, 1000, 2000 Hz: mild for 21–40 dB, moderate for 41–70 dB, severe for 71–95 dB and profound for >95 dB.

2.3. Molecular analysis

2.3.1. DNA preparation

Genomic DNA was extracted from peripheral blood of all

patients and other available members of the families as well as 50 normal control subjects using a standard phenol chloroform procedure. DNA quantification and quality analyses were performed by spectrophotometry (UNICO 2100, USA) and agarose gel electrophoresis using routine procedures.

2.3.2. Genotyping microsatellite markers

Selection of DFNB21 short tandem repeat (STR) markers linked to *TECTA* and their primers were selected based on their physical distance at NCBI map viewer (<http://www.ncbi.nlm.nih.gov/mapview>). Markers used in this study included: D11S1774, D11S925 and, D11S4089 at the upstream of the *TECTA* gene and D11S4167, D11S1345 and D11S4094 which were downstream markers. The heterozygosity of these markers and their positions relative to this gene is shown in Table 1. Slink value for Ahv-9 family was calculated using FastSlink (version 2.51) option of EasyLinkage plus (version 5.05) software. Analysis of two-point and multi-point LOD scores were done by SuperLink (version 1.5), and GenHunter (version 2.1r5) options of EasyLinkage (version 5.05), respectively [17]. Haplotypes for STR markers were reconstructed using GeneHunter and were visualized by HaploPainter software (version 029.5) [18]. For calculations of LOD scores, autosomal recessive pattern of inheritance, complete penetrance and no phenocopy, equal recombination for male and female allele frequency of markers and disease allele frequency of 0.001 were assumed [19].

2.3.3. Linkage analysis

The following amplification conditions were considered for STR markers, with some modifications for each amplicon. Reactions were carried out in a 25 ml volume containing 10 ml Taq x master mix red (Ampliqon, Hamburg, Germany), 0.5 ml of each of the primers (10 pM), 2 ml DNA (50 ng), 10 ml ddH₂O. The PCR program for STR amplification was as follows: one cycle of 95 C for 3 min (initial denaturation), 30 cycles of 94 C for 30 s (denaturation), 58 C for 30s (annealing), 72 C for 30s (extension) and one cycle for the final extension at 72C for 5 min. The amplification products were analyzed by conventional polyacrylamide gel electrophoresis and silver staining.

2.3.4. DNA sequencing of *TECTA*

Primers for all the 23 coding exons, exon–intron boundaries, and promoter of *TECTA* were designed by primer3ver.0.4.0 (<http://frodo.wi.mit.edu/primer3/>) and Oligover.6.7.1.0 (National Biosciences Inc.), (Table 2). Amplifications of all the exons and the promoter of *TECTA* were carried out according to the following program, with some modifications for different amplicons: one cycle of 95 C for 3 min (initial denaturation), 30 cycles of 94 C for 30 s (denaturation), 58 C for 30 s (annealing), 72 C for 30 s (extension) and one cycle for the final extension at 72 C for 5 min. DNA sequencing of the PCR products were carried out bidirectionally on an ABI 3730XL automated sequencer (Applied Biosystems) (MacroGen, South Korea) using the same primers.

Table 1
Heterozygosity and position of selected markers for the DFNB21 locus.

Marker name	HZ (%)	Forward primer (5' → 3')	Reverse primer (5' → 3')	PCR product size (bp)	Physical position (bp)
D11S1774	57	CAAAAAGGCTTGCGGTT	GGGCATTCCCATGCTCA	206	120,820,059–120,820,264
D11S925	85	TTAGACCATTTATGGGGGCAA	AGAACCAAGGTCGTAAGTCCTG	173	120,828,211–120,828,546
D11S4089	75	TAATCAAAGGCTGTAGTGAATTGG	ATTCTAGTTCCTCATAAACACTG	199	120,989,623–120,989,921
D11S4167	77	TCCCCACCTGCCTGAG	TGTTGACCAAAATGTTGTTATGC	106	122,037,647–122,037,752
D11S1345	—	AGCTAAGATGTGCCACAGTAA	TCAGTGCTGAGCCCATATAT	237	122,427,077–122,427,313
D11S4094	80	CTCAAAGAACAGCCAGTCA	GGAGTCGGGGAATTTCTAA	196	123,176,594–123,176,789

HZ: Heterozygosity.

Table 2
TECTA primers for amplification of the promoter and 23 exons.

Promoter/Exon	Forward primer (5' → 3')	Reverse primer (5' → 3')	PCR product length (bp)
Promoter	CTACAGAACAGGTTAGTGACAT	GCCAGAGTAACAGAACACCA	1192
1	TTTTGATGGTCTTGGGATTAGC	AAGTGGGAATATAAGTAAACCTC	355
2	AGGATGAATGACAGGGCAGTA	CATGAAATCTCAGAACTTGTCTG	418
3	CATACAGTTAGGCGAATGTCT	AACTGACACTCCTTAGCTCTA	486
4–5	GGGGAGTGCAAGTCATGAG	CATTACCCAGCGGAGAGAT	817
6	CTAACAGGGTCTTACGTGGA	GAGACAAACAGCAGAACCACT	627
7	TCTGGAGTGTGAGTTGCTT	TTGGGTCACAAGTCTTAACAA	774
8	CACCTCTAGGAACCTAATGATCCA	TTCTAAAATCCACTCATAGGCAG	809
9	TCGGTTGGTATCTTTTGGGGTT	AGGACACATCAGACTAGGGTA	869
10	CCGTCTTCTCCTTGACCACC	TGCCTCCTAACTTGGTGTAT	857
11	TGCATTCAACCTGCTCAAACTT	TCTATTGTGAACCTTTGTGCGT	987
12	TGAGAAACCCTCCTCGGTG	CCATTCCCCTGCCACGAACA	393
13	GACGAGGGGGACTGGGCTTTC	AGCAACAATCTATCCTACAACCT	569
14	GCTGCAGGGACAGAAATGGAGTC	ACGAAATTAAGTGGGCTCTCTGAT	608
15	CCATCATTCCTATTTACCTCAC	CTCACAGCTCTACTATCCA	679
16	CTGCCATCTGACCATTTCAT	GCATCAAGGTCGTTCCCA	302
17	CTAAAGGAGAATGGAATGGGA	CTAAGGAGCATATTTCTAAGCAG	411
18	TCCATGCTGATTATACCTGGCT	AGGGCATCAAGACAAACGCT	397
19	CCTTAGACTTTGCTACTACGTG	ATGCAAAATCTGGCCTTGTA	491
20	GAGTGATAATAAATGACAAGCCC	GTTTTCTCTACTGTCCATCAA	487
21	TTTTGATGGTCTTGGGATTAGC	AAGTGGGAATATAAGTAAACCTC	765
22	CCTCTCTTTTAAAGCCCTCCC	GAGTTTTCGAGGGAGTTGC	317
23	GCAAAGATGTCTGATCCCTA	GGCACACACTTTTATCCTACA	248

2.4. Pathogenicity investigation

Using the newly published criteria for pathogenicity of variants [20], several lines of evidence were provided to confirm the pathogenicity of the variant. These included 1) The inheritance pattern 2) using *in silico* prediction software tools (ConSurf) and search within population databases such as 1000 genomes, exome aggregates and exome variant server, dbSNP and dbVar 3) The length of the predicted truncated protein 4) Co-segregation of the variant with deafness in the family and 5) investigation of 100 chromosomes of healthy ethnically-matched controls.

2.5. In silico investigation

Conservation scores for each amino acid in TECTA were acquired from the ConSurf Server (<http://conseq.tau.ac.il/>). Any conservation score greater than 1 standard deviation above the mean for the TECTA amino acid sequence was regarded as highly conserved.

2.6. Co-segregation analysis and investigation of healthy controls

The presence of homozygous variant in affected individuals its absence in the non-affected siblings (co-segregation) of the family and absence of the novel variant in the normal control individuals were used to further confirm the pathogenicity of the variant. For investigation of pathogenicity of c.734G > T in exon 5 was investigated by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The restriction enzyme *FspBI* (*Bfal*) was selected for RFLP analysis of exon 5, using *Nebcutter* (version 2.0 <http://tools.neb.com/NEBcutter2>). After amplification of exon 5 in 50 (100 chromosomes) ethnically matched controls and all available members of Avh-9 family, the amplicons were digested and loaded on 8% PAGE at 35 mA for 2 h, followed by visualization using silver staining. After digestion, the normal allele should have 435 bp and 216 bp but the mutant allele should have 248, 216 and 187 bp fragments. Therefore, normal and mutant alleles are different for 435 bp fragment which is not digested in the normal allele but is digested to two smaller 216 and 187 bp fragments in the mutant allele.

3. Results

3.1. Phenotype evaluation results

Totally, 6 Out of the 25 multiplex deaf families negative for *GJB2* mutations, had moderate-to-severe phenotype and one of them linked to DFNB21 (Ahv-9 family). Marriage in the Ahv-9 family had occurred between second cousins (subjects 7 and 8). The pedigree of the family Ahv-9, including three generations and with 15 members, was consistent with an autosomal recessive inheritance pattern. Based on medical records, HL in the affected subjects was prelingual, symmetric, and moderate to severe across all frequencies but more pronounced in mid-frequencies. All heterozygous carriers had normal hearing for sex and age.

3.2. Molecular analysis results

After DFNB21 STR marker genotyping, haplotypes were drawn. Homozygosity mapping was performed using six markers linked to the DFNB21 locus on 25 Iranian families negative for *GJB2* mutations. Nine members from family Ahv-9 were selected for linkage analysis. In the Ahv-9 family, we detected homozygosity in all patients. Fig. 11 depicts the haplotype for this family. Slink, Two-point and multi-point LOD scores were 2.77, 2.32 and 2.49, respectively. In order to confirm the result of linkage analysis and to identify the pathogenic variant, all the 23 exons and promoter of the TECTA gene were sequenced. The results revealed a novel variant (c.734G > A) in exon 5 of TECTA gene (the entactin G1 domain of the protein) in the patients (Fig. 1.II). This nonsense variant results in a premature stop codon (p.W245X). It leads to a truncated protein and the affected residue is highly conserved across species (Fig. 1.III). It segregated in homozygous state in all the affected family members. The variant was not found in 50 (100 chromosomes) ethnically matched healthy controls. Several SNPs were also found which are listed in Table 3. Based on the American College of Medical Genetics and Genomics (ACMG)- Association for Molecular Pathology (AMP) guideline this variant is very strong pathogenic (PVS1). (Pathogenic criterion is weighted as very strong (PVS1), strong (PS1–4); moderate (PM1–6), or supporting (PP1–5), and each benign criterion is weighted as stand-alone (BA1), strong (BS1–4), or supporting (BP1–6)).

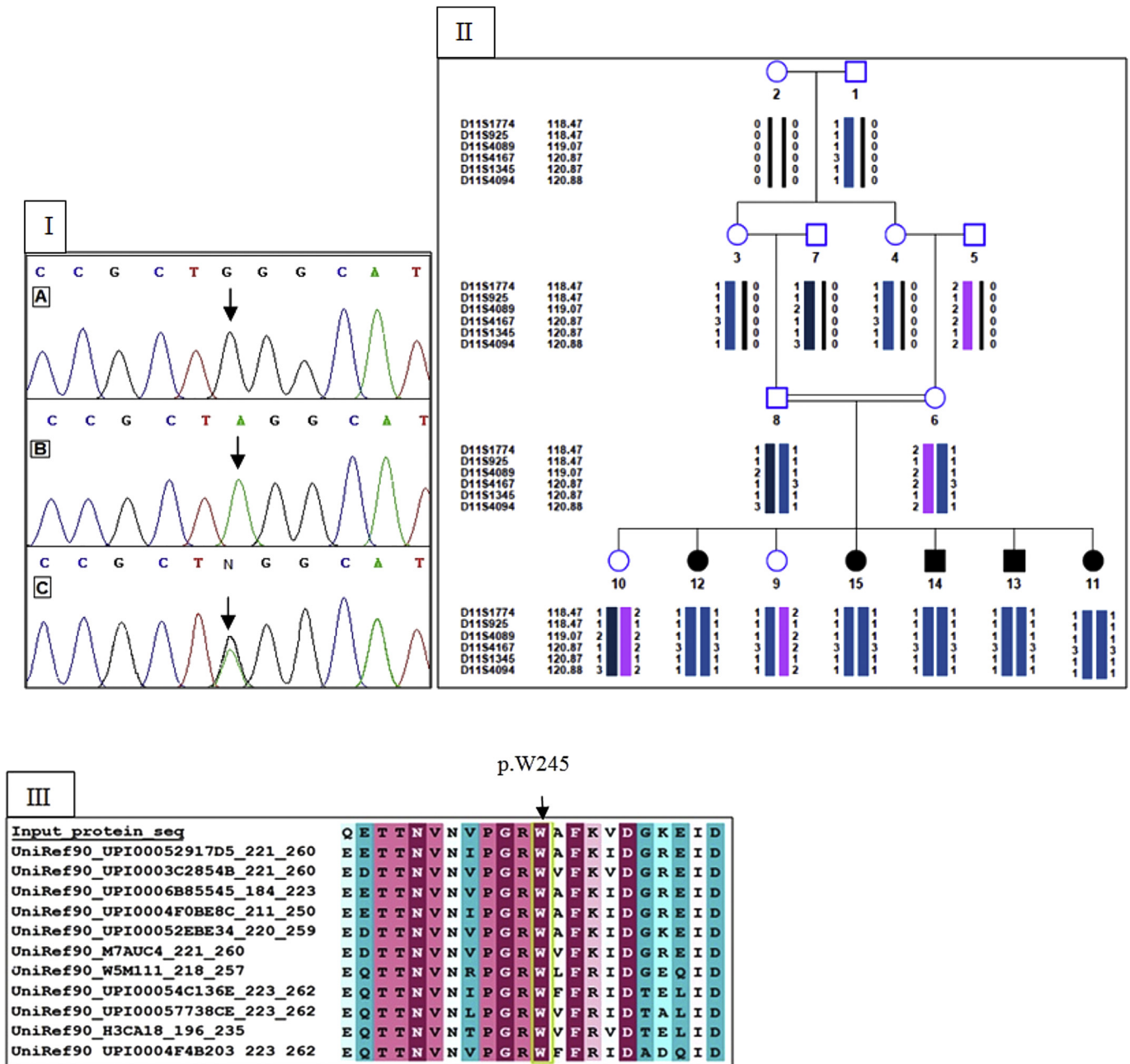


Fig. 1. Linkage to DFNB21 and *TECTA* mutations. (I) Family Ahv-9 shows co-segregation of nonsyndromic recessive deafness with DFNB21 linked markers on chromosome 11q. Deaf subjects 11, 12, 13, 14 and 15 show homozygosity for markers linked to DFNB21. They are affected and homozygous. (II) Sequence electropherogram shows the c.734G > A mutation found in this family. Partial *TECTA* sequences are shown wild type (Homozygous) (A), affected (B) and carrier (Heterozygous) (C). (III) Pathogenic variants (c.734G > A) occur at a highly conserved position (p.W245). The corresponding DNA sequences appear in green box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, 6 out of the 25 studied families with ARNSHL and negative for *GJB2* mutations showed moderate to severe HL. A panel of genes including *TECTA* (DFNB21) was investigated in the families (data not shown). Linkage to DFNB21 was only seen in Ahv-9 family. Upon sequencing of the gene in affected members the homozygous c.734G > A variant was identified. This nonsense variant (p.W245X) leads to a truncated protein with only 82 residues which has lost most of its functional domains including terminal part of ENTG1 domain, four von Willebrand type D (vWFD V1, V2, V3, V4) domain and C-terminal zonapellucida (ZP) domain,

Table 3

TECTA variants identified in family Ahv-9.

Exon/Intron	Nucleotide variation (NM_138691.2)	Reference SNP (rs) number
Exon 5	c.734G > A	p.W245*
Intron 6	c.1197 + 4 A > C	—
Exon 7	c.1485A > G	SNP rs536069 (A/G)
Exon 8	c.2256 C > T	SNP rs57691032 (C/T)
Exon 9	c.2805T > C	SNP rs586473 (T/C)
Exon 15	c.5171G > A	SNP rs526433 (G/A)
Intron 21	c.6250 + 314C > T	—

* indicate the stop codon.

Table 4
Mutations identified in the *TECTA* gene and their audiometric profile.

Exon	Mutation	Protein change	Domain	Time of onset	Frequencies	Family origin	Reference
5	IVS9 + 1 G > A	p.248× ^a	ENT	Prelingual	All frequencies	Lebanese	[12]
3	c.266delT	p.Leu89Arg ^a 34	ENT	Prelingual	Mild	Iranian	[23]
5	649insC	—	ENT	Prelingual	Mild	Iranian	[23]
5	c.651dup C	p.Asn218GlnFS ^a 31	ENT	Prelingual	All frequencies	Iranian	[14]
5	c.734G > A	p.W245 ^a	ENT	Prelingual	All frequencies	Iranian	This study
9	c.2941 + 1 G	—	ZA	Prelingual	All frequencies	Iranian	[12]
9–10	9.6 Kb	del	ZA	Prelingual	Mild	Iranian	[23]
15	c.5211 C > A	p.Tyr1737 ^a	ZA	Prelingual	Mild	Iranian	[23]
15	c.5210A > G	p.Y1737C	C8 domain	Prelingual	Mild	Iranian	[25]
20	c.6037delG	p.2018× ^a	ZP	Prelingual	All frequencies	Pakistani	[14]
21	c. 6203–6218 del	p.lys 2068Arg ^a 38	ZP	Prelingual	All frequencies	Iranian	[26]

ZP: Zonapellucida domain, ZA: Zonadhesin domain.

^a Protein may not be formed due to nonsense mediated decay.

result in, this would be functional null allele.

Most of the dominant *TECTA* (DFNA8/12) mutations been reported in ZP and WF domains, which are associated with stable and post-lingual progressive [21]. However, recessive mutations of *TECTA* (DFNA21) have been reported in all domains, and are usually linked to prelingual stable HL. Interestingly, the substituted nucleotide found in this study (c.734G > A) resides in the ENTG1 domain which is conserved among human, rat, and chicken alpha-tectorin (Fig. 1.III). The homology among these three orthologs is over 70%.

Based on the ACMG-CAP guideline [20]: 1) This variant is nonsense (p.W245×), tryptophan codon replacement with stop codon at residue 245 ((PVS1). 2) The homozygosity of this variant conforms to the autosomal recessive pattern of inheritance (PM3). Co-segregates with deafness: heterozygous carriers do not show the deafness phenotype but all the homozygous individuals were affected (PS4). 3) This variant was not found in population databases including: exome sequence variant server (<http://evs.gs.washington.edu/EVS> and <http://browser.1000genomes.org>), databases SNP (<http://www.ncbi.nlm.nih.gov/snp>) and databases variant (<http://www.ncbi.nlm.nih.gov/dbvar>) (PM2). 4) These domains which are affected in this way are important because are conserved domain (the end point of ENTG1, four von Willebrand type D and ZP domain are omitted) (Fig. 1.III) (PM1) 5) The c.734G > A variant is predicted to result in a premature stop codon in the amino acid position 245 (p.W245×) in the fifth exon with production of a truncated protein (PSV1). 6) It was not found in ethnically matched healthy controls (PS4).

According to these evidences (2PVS1+ PM3+ PS4+ PM2+ PM1) and based on the guideline ACMG-CAP [20], this variant meets the criteria of being categorized as very strong pathogenic.

In the mouse mutant homozygous for a targeted deletion in the ENTG1 domain of alpha-tectorin protein, the tectorial membrane lacks its non-collagenous matrix and is detached from the surface of the organ of Corti, leading to HL. The heterozygous mutant has a normal tectorial membrane and normal hearing [22]. An alteration of the α -tectorin protein is likely to disrupt the normal structure of the extracellular matrix of the tectorial membrane, in consequence, leading to inefficient transmission of sound [23]. The frequency of *TECTA* involvement (4%), we have found in the studied population (in *GJB2* negative ARNSHL families) conforms to the findings already reported in the Iranian population. For example, *TECTA* was involved in 10 out of 242 families (4.13%) *GJB2* negative ARNSHL families [6]. So far, no reports of ARNSHL of *TECTA* gene (DFNB21) has been published in the Caucasians and all reports of this mutation are limited to the Middle East including Lebanon [21] and Pakistan [14] populations (Table 4). Different studies have shown that inactivating and non-truncating mutations of *TECTA* have been associated with autosomal recessive and autosomal dominant

forms of HL, respectively [24]. Our results and other former studies emphasize that the mutations in the *TECTA* gene are among most common causes of ARNSHL in the Iranian population. All mutations found in the Iranian population are truncating loss-of-function mutations leading to a characteristic audioprofile that is recognizable as moderate to severe HL across all frequencies, but most pronounced in the mid-frequency range [14,24].

In conclusion, we have identified a novel pathogenic variant in an Iranian family. Our study, together with the data of several other studies, suggests that in deafness diagnostics mutation analysis of *TECTA* should be prioritized in the screening of families segregating ARNSHL with moderate to severe audioprofile.

Conflict of interest

Authors declare no conflicts of interest.

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